Effects of sub-lethal and chronic lead concentrations on blood and liver ALA-D activity and hematological parameters in Nile tilapia

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Liver and blood δ-aminolevulinic acid dehydratase (ALA-D) inhibition by exposure to sub-lethal lead concentrations over time in Nile tilapia (Oreochromis niloticus) were investigated. All three lead concentrations (1 mg kg \(^{-1}\), 10 mg kg \(^{-1}\) and 100 mg kg \(^{-1}\)) significantly inhibited ALA-D activity in blood (319 ± 29.2; 180 ± 14.6 and 172 ± 19 μmol h \(^{-1}\) L \(^{-1}\) respectively) and liver (302 ± 5.84; 201 ± 41.4 and 93 ± 22.1 μmol h \(^{-1}\) L \(^{-1}\)) 24 h after injection relative to controls (blood: 597 ± 37.0 μmol h \(^{-1}\) L \(^{-1}\); liver: 376 ± 23.1 μmol h \(^{-1}\) L \(^{-1}\)). Blood ALA-D was greatly inhibited in all but the highest lead dose. Fish were then exposed to 1 mg kg \(^{-1}\) lead for 9 days, and presented short-term hyperglycemia, decreased hemoglobin and hematocrit values and time-dependent blood ALA-D activity inhibition, corroborating blood ALA-D activity as being more suitable for investigating lead effects, showing dose and time-dependent ALA-D inhibition after lead exposure. The results of the present study also demonstrated that fish size affects blood ALA-D activity, as fish from the 24-h assay, which were slightly smaller (approximately 200 g), showed higher ALA-D inhibition in response to lead exposure when compared to the fish from the 9-day assay (approximately 500 g). Thus, fish size should always be taken into account both in the field and in laboratory settings, and efforts should be made to obtain uniform fish size samples for biomarker studies.

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1. Introduction

Lead is a major environmental contaminant, and, in aquatic ecosystems, exposure can cause toxic effects such as hematological, renal, reproductive, behavioral and neurological deleterious effects in fish, which can eventually lead to death (Atli and Canli, 2007; Burden et al., 1998; Fernández et al., 2015; Miloskovic et al., 2013). One of the most recognized lead effects is the inhibition of the enzyme δ-aminolevulinic acid dehydratase (ALA-D).

ALA-D is a key enzyme in the Heme biosynthesis pathway. It asymmetrically condenses two molecules of delta aminolevulinic acid (ALA), forming a porphobilinogen (PBG) molecule. This enzyme requires zinc co-factors for proper function, but, as it is rich in sulfhydryl groups, it also shows affinity to other metals, which then displace Zn from the molecule active site, resulting in an identical molecular structure but improper functioning and, consequently, ALA-D inhibition (Fernández et al., 2015). The affinity of ALA-D for lead is 25 times higher than for Zn, which makes this enzyme a selective and fast-responding biomarker of lead exposure and bioavailability (Lombardi et al., 2010)(Fernández et al., 2015). Usually, biomonitoring studies, especially in the aquatic environment, are conducted by investigating liver tissue biomarkers, since this is the main detoxifying organ of the body. In the specific case of lead, however, blood has been preferred in these types of studies, since it is the main target for lead toxicity and can be collected without sacrificing the animals, although some studies comparing both matrices have also been conducted (Campana et al., 2003; Schmitt et al., 2002). In fact, in many cases, although less specific, several hematological parameters in fish have been shown to be potential effect biomarkers such as hematocrit, hemoglobin, protein and glucose levels (van der Oost et al., 2003), and studies have strongly suggested that the presence of metals in the aquatic environment has a strong influence on these parameters in several fish species (Vinodhini and Narayanan, 2007; Burden et al., 1998; Fernández et al., 2015).
2.2.1. 24-h exposure assay

2.2. Lead exposure experiments

Ethics Committee on the use of animals of the Oswaldo Cruz Foundation (Fiocruz) (Approval number L 0033/08).

2. Methodology

2.1. Fish subjects

Fish were transported in plastic sacks filled with oxygen-saturated water, separated and acclimatized for 10 days before experimentation, in plastic tanks containing 50 L of aerated water maintained at 23 ± 2 °C, previously filtered with cellulose and charcoal filters. Water pH was maintained at 6.4 ± 0.2 and changed every two days. A maximum of 600 g of fish was maintained per tank. The lead concentrations tested herein were extrapolated from rat intraperitoneal LD50 values in response to exposure to lead tetracarbamate (90 mg kg⁻¹) and are environmentally relevant (IPCS, 1994; NIOSH, 1994). All punctures for injection or blood collection were made with 29 gauge needles (0.3366 mm of nominal diameter). This study was carried out according to the manufacturer’s instructions. The assay was carried out in strict accordance to international guidelines and with the approval of Ethics Committee on the use of animals of the Oswaldo Cruz Foundation (Fiocruz) (Approval number L 0033/08).

2.2. Lead exposure experiments

2.2.2. Nine-day exposure assay

The results from the previous assay allowed for the selection of the most suitable concentration for evaluation of lead-exposure effects during longer exposure periods, of 1 mg/kg⁻¹. Fish weighing approximately 500 g were blood sampled (day 0; n=8) and then divided into two groups. The control fish (n=3) were i.p. injected as previously described, while the lead-exposed animals (n=5) were i.p. injected with a single-dose (1 mL) of the isotonic glucose solution containing 1 mg/kg⁻¹ of lead acetate. Blood samples were then obtained daily during nine days, at the same time. Several hematological parameters were also investigated during this assay, described in the next section.

2.3. Glucose determinations

Blood samples were centrifuged (10,000g for 5 min) and 10 μL of plasma were used for glucose quantification with an enzymatic commercial kit (Bioclin) with endpoint reading, according to the manufacturer’s instructions. Spectrophotometric readings were performed, in triplicate, after 20 minutes at 505 nm, according to the manufacturer’s instructions.

2.4. Hematocrit determinations

Heparinized capillary tubes 75 mm in length and with inner diameter of 1.0 mm (Perfecta) were used to determine the hematocrit, in triplicate. The capillaries containing blood were centrifuged for 10 min (model Q-22M microhematocrit Quimis centrifuge) and readings were taken immediately after centrifugation through a hematocrit reader chart.

2.5. Hemoglobin determinations

Hemoglobin was assayed in triplicate, using a commercial kit (Bioclin) based on the use of a Drabkin solution and a commercial standard with 11.3 g·dL⁻¹ of stable hemoglobin. The assay was carried out according to the manufacturer’s instructions.
2.6. ALA-D enzymatic assays

ALA-D activity in blood and liver supernatants were assayed in triplicate by a protocol based on methodologies described elsewhere (Sakai et al., 1980; Wigfield and Farant, 1981). This method is based on the determination of the enzymatic ALA-D activity through the spectrophotometric quantification of the amount of porphobilinogen formed during an hour of incubation with the enzyme substrate ALA. Briefly, cell lysis was achieved by adding water and Triton X-100 (0.05%, each sample. After five minutes, a 50 mmol L\(^{-1}\) potassium phosphate buffer solution, pH 6.4 and 4 mmol L\(^{-1}\) ALA (final concentrations) were added to the lysed samples. After one hour at 37 °C the reaction was stopped by the addition of trichloroacetic acid/mercury chloride (123 mmol L\(^{-1}\)). The graph was constructed using the GraphPad software package. The IC50 was determined by linear regression (% inhibition in relation to the mean of controls (represented as 100%)).

All exposed animals showed significant decreases in blood ALA-D activity. Liver ALA-D activity, however, was significantly reduced only in the 10 mg and 100 mg exposed groups, which suggests a less sensitive response of this enzyme in liver. This corroborates other studies that have reported significant differences regarding blood and liver ALA-D activity, with blood activity being significantly higher than liver activity at short Pb-exposure periods (Campana et al., 2003). For example, an in vitro ALA-D study conducted with spotted Pimelodus (Pimelodus maculatus) also found higher inhibition of blood ALA-D activity when compared to hepatic ALA-D (Rodrigues et al., 1989). It should also be noted that, besides blood ALA-D susceptibility to lead, this matrix may also act as a Pb reservoir, leading to subsequent ALA-D inhibition (Alves et al., 2006; Brumbaugh et al., 2005).

3.2. Lead IC50 for ALA-D inhibition in Nile tilapia blood and liver

The linear regression and the IC50 calculation for ALA-D inhibition by lead exposure are displayed in Fig. 2. Each point was calculated with the results displayed in this figure and the bars represent ln (1 Standard Deviation). Results are expressed in percentage in relation to the mean of controls (represented as 100%).

3.3. 24-h lead exposure assay

No statistically significant difference (\(p > 0.05\)) was observed for any biometric parameters (length and weight) between the controls and the lead-exposed fish.

Regarding ALA-D activities, the mean ALA-D activity in the blood of control animals was significantly higher (\(p < 0.05\)) when compared to mean ALA-D blood activity of the fish treated with the three lead acetate doses. Mean hepatic ALA-D activity of controls, on the other hand, was significantly higher (\(p < 0.05\)) than animals exposed to 10 and 100 mg lead acetate doses, with greater inhibition observed at 100 mg (Fig. 1). Blood ALA-D activity inhibition of the animals exposed to 1 mg kg\(^{-1}\) and 10 mg kg\(^{-1}\) lead were significantly higher, 47% and 70%, respectively, than hepatic ALA-D inhibition, of 20% and 47%, respectively. At 100 mg kg\(^{-1}\), both blood (71%) and liver (75%) showed similar inhibitions.

In the 24 h lead exposure assay, ALA-D activity was assayed in blood and liver supernatants of the three lead acetate groups (Fig. 1). A molar extinction coefficient (\(e\)) of 0.062 nmol cm\(^{-1}\) L\(^{-1}\) was considered for enzyme calculations.

Statistical analyses

Analyses of biometric and enzymatic results were performed using the GraphPad Prism 5.0 version 5.0 software package. Data normality was tested using the Shapiro-Wilk W test prior to additional statistical analyses. As the data showed a non-normal distribution, non-parametric tests (Mann-Whitney) were applied.

Fig. 1. ALA-D levels in blood (A) and liver (B) of tilapia controls and those exposed to 1, 10 and 100 mg kg\(^{-1}\) of lead. Blood ALA-D activity is expressed as \(\mu\)mol PBG h\(^{-1}\) L\(^{-1}\), while liver ALA-D activity is expressed as \(\mu\)mol PBG h\(^{-1}\) kg\(^{-1}\). Different symbols indicates statistical differences between treatments.
3.3. Nine-day assay

3.3.1. Glucose levels

Lead-treated fish presented a non-significant, short-term, hyperglycemia trend in the 1 mg lead-treated fish, 24 h after injection, with a subsequent return to normal values throughout the nine evaluation days (Fig. 3).

These results corroborate previous reports that indicate different metals as glycemia-stimulants in fish. For example, tilapia exposed to lead 0.05 mg L\(^{-1}\) developed hyperglycemia associated to cortisol increases (Firat et al., 2011), while a short increase in tilapia blood glucose concentrations following cadmium exposure has also been reported (Lin et al., 2011). This has been postulated to be caused by the induction of glycogen phosphorylase activity. Curimbatá specimens (\textit{Prochilodus lineatus}) exposed to lead nitrate for 96 h and juvenile rockfish (\textit{Sebastes schlegelii}) exposed to lead for 4 weeks also displayed classic hyperglycemia associated with lowered plasma lipids and proteins (Kim and Kang, 2015; Martinez et al., 2004).

3.3.2. Hematocrit and hemoglobin levels

A small, non-significant, decrease of hematocrit values in the controls was observed after the fifth day and may be related to blood withdrawal. Exposed fish, however, showed earlier and statistically significant decreases in hematocrit values from the second day onwards (Fig. 4).

Hemoglobin levels in the controls showed no significant differences during the entire 9-day assay, while exposed fish showed a significant decrease only on the ninth day (Fig. 5).

These results also corroborate previous reports in the literature for both tilapia and other fish species experimentally exposed to lead and other metals. For example, Mozambique tilapia semistatically exposed to low (L) (0.5 mg L\(^{-1}\)), medium (M) (2.5 mg L\(^{-1}\)), and high (H) (5 mg L\(^{-1}\)) lead concentrations for 14 days showed significant decreases in the M and H groups for red blood cell (RBC) count, hematocrit (Hct) ratio and hemoglobin levels, while the RBC was significantly increased in the L group compared to control group (Kaya et al., 2013). In another lead exposure study, conducted with \textit{Tilapia zillii}, both the hemoglobin content and RBC of the exposed fish showed significant decreases.
from control values (Ghazaly, 1991). In a study conducted with trout (Onchorhynchus mykiss) exposed for 72 h to lead demonstrated significantly decreased hemoglobin and hematocrit values (Ates et al., 2008), while the same was observed for North African Catfish (Clarias gariepinus) exposed for 96 h (Adeyemo, 2007) and juvenile rockfish (S. schlegelii) exposed for 4 weeks (Kim and Kang, 2015). In another study, common carp (Cyprinus carpio) exposed to a mixture of metals (Cd, Pb, Cr, Ni) for 32 days showed significantly elevated red blood cell, glucose and total cholesterol concentrations in comparison to controls (Vinodhini and Narayanan, 2009), while experiments conducted with Indian major carp (Labeo rohita) verified that exposure to hexavalent chromium induced significant decreases in total erythrocyte count and hemoglobin and glycogen levels, indicating metal induced cumulative deleterious effects (Vutukuru, 2005).

Red blood cell membranes are a potential target for a lead-initiated lipid peroxidation process leading to morphology alterations and osmotic change (Ercal et al., 2001). Indeed abnormal erythrocyte morphology, hemolysis and erythrocyte fragility in rats chronically treated with 15 mmol L\(^{-1}\) of lead acetate has been previously reported (Ahayauach et al., 2013), as well in Nile Tilapia exposed to lead chloride at 0.1 mg L\(^{-1}\) (Çoğun and Şahin, 2013). An in vitro assay study also reported lead dose-dependent erythrocyte hemolysis (Mrugeshi et al., 2011). Therefore the hematocrit and hemoglobin concentration reductions observed in the present study may be a consequence of lead-induced erythrocyte hemolysis over the exposure period.

More evident hematological alterations in the last days of experimental Pb exposure over a certain timeframe have been related in other fish species, such as in a study conducted with Pb-treated toadfish (Halobatrachus didactylus) that indicated some hematological alterations related to hemoglobin synthesis and kidney disturbances (anemia and hemoglobin decreases, among others). The authors postulate that these hematological pathologies may be related to Pb increases and to histopathological alterations in the kidney (especially in renal hematopoietic tissue

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**Fig. 4.** Hematocrit levels of tilapia controls (A) and injected intraperitoneally with 1 mg kg\(^{-1}\) lead (B). Each symbol represents the result for one individual animal. Daily fish group means was used for comparison between days. An asterisk (*) indicates statistical difference between daily group mean in comparison to day 0.

**Fig. 5.** Hemoglobin levels of tilapia controls (A) and injected intraperitoneally with 1 mg kg\(^{-1}\) lead (B). Each symbol represents the result for one individual animal. Daily fish group means was used for comparison between days. An asterisk (*) indicates statistical difference between daily group mean in comparison to day 0.
and renal tubules), since other studies regarding metal exposure in fish show evident degeneration of tissues at the histological level (Campana et al., 2003).

3.3.3. ALA-D activity

When compared to day zero, both the control and lead-treated groups showed ALA-D activity decreases in the days following the injection. However, in comparison to the first day, control groups presented ALA-D inhibition on the fifth and seventh days, with enzyme activity recovery on the ninth day. Lead-exposed fish presented a more pronounced ALA-D activity decrease, with inhibition on all but the fifth day when compared to the first day (Fig. 6).

Both the control and lead treated animals presented ALA-D activity inhibition 24 h after injection that may be related to injection stress. However, the ALA-D activity decrease in the control group at the fifth and seventh day may be related to blood withdrawal with subsequent restoration to normal values on the ninth day. This seems to confirm that gradual tissue lead incorporation after parenteral exposure enhances lead bioavailability over test periods with consequent declines in ALA-D activity (Varni et al., 2001).

Suppression of ALA-D over time has been described for other fish species, such as carps (100 μg kg⁻¹) lead exposure, which showed increasing ALA-D inhibition during prolonged exposure periods (Nakagawa et al., 1995). A time-dependent ALA-D activity decrease was also reported for brown trout after eleven days of exposure to a lead-impacted runoff stream (Heier et al., 2009).

The results of the present study regarding the occurrence of a significant negative correlation between Pb concentrations and blood ALA-D activity are also in accordance with previous studies with other fish species, such as Curimbatá (P. lineatus) specimens exposed to lead in the laboratory (Martinez et al., 2004) and several fish species sampled from impacted sites, including common carp (C. carpio), largemouth bass (Micropterus salmoides), channel catfish (Ictalurus punctatus), largescale stoneroller (Campostoma oligolepis), longear sunfish (Lepomis megalotis) and northern hog sucker (Hypentelium nigrican) (Schmitt et al., 2007) curimbatá (P. lineatus) (Lombardi et al., 2010) and red mullet (Mullus barbatus) (Fernández et al., 2015).

However, it is worth noting that results regarding ALA-D activities in lead-exposed fish have been controversial. For example, while many studies have indicated that ALA-D is a good indicator of lead exposure in fish, other studies did not find any significant differences between controls and lead-exposed fish, such as in the study conducted with toadfish specimens (H. didactylus) that concluded that ALA-D blood levels are not suitable as endpoint measures of lead pollution in this species, since the authors were not able to demonstrate any direct dose–response relationship between injected lead concentrations and the enzymatic ALA-D activity (Campana et al., 2003). In fact, the authors of that study hypothesize that different uptake routes and different taxa could be responsible for the wide variability of results on the effect of lead on ALA-D activity. Thus, the results of the present study further highlight the importance of further investigations with regard to establishing good bioindicators and biomarkers among different teleost species for environmental monitoring programs (Lopes et al., 2014).

When comparing both assays, fish from the 24-h, assay showed higher ALA-D inhibition in response to lead exposure when compared to the fish from the 9-day assay. This may be due to the different sizes of both groups (200 g and 500 g, respectively). This has been described previously with regard to metal effects, in which fish presented size-dependent ALA-D inhibition, with a higher degree of inhibition observed in smaller fish (Burden et al., 1998; Kanak et al., 2014). Direct relationships between fish size and hematological parameters, such as hemoglobin and hematocrit values, have also been reported when different fish-sized groups were compared, which makes sense since quantity and quality of blood tends to increase with size (Gabriel et al., 2011; Jawad et al., 2004). Thus, fish size should always be taken into account both in the field and in laboratory setups, and efforts should be made to obtain homogenous samples regarding fish size.

4. Conclusions

The results in tilapia from the present study corroborate blood ALA-D activity as being more suitable for investigating lead effects, showing dose and time-dependent ALA-D inhibition after lead exposure, coupled to other changes in hematological parameters, such as hyperglycemia and hematocrit and hemoglobin decreases. These blood parameters, when measured in conjunction with biomarkers with more specific response patterns (such as cytochrome P450 and acetylcholinesterase activities) may prove to be important biomarkers in environmental risk assessments using Nile tilapia as a sentinel species of environmental quality.
results also highlight the importance of further studies with regard to the establishment of adequate bioindicators and biomarkers among different teleost species for practical use in environmental monitoring and risk assessment programs.

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